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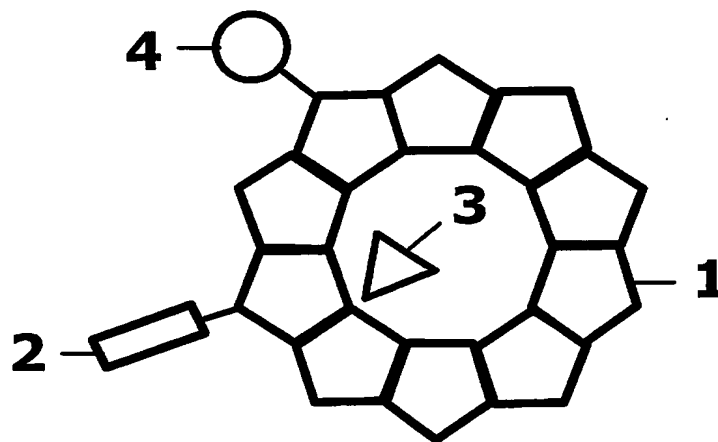
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ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: NANOPARTICLE FOR BIOAFFINITY ASSAYS



(57) Abstract: Thus this invention relates to a nanoparticle, useful for bioaffinity assays. The nanoparticle has a self-assembling shell built up of several protein and/or peptide subunits, which protein and/or peptide subunits can be of one or several different types, assembled in an organized manner to form the shell having an inner surface facing the inside and an outer surface facing the outside of said particle. One or several of the types of subunits have one or several first binding moieties per type of subunit with the binding moiety facing the outside of the particle for binding of any specific ligand binding protein; and the particle contains within its shell a marker and/or one or several of the types of subunits have one or several second binding moieties per type of subunit with the binding moiety facing the inside and/or the outside of the particle for binding a marker; and the marker or markers enables detection of the particle. Characteristic for the nanoparticle is that the shell of the nanoparticle is a recombinant apoferritin or an apoferritin-like particle. This invention also relates to a bioaffinity assays using the nanoparticle. This invention further relates to a kit for bioaffinity assays comprising the nanoparticle.

NANOPARTICLE FOR BIOAFFINITY ASSAYS

FIELD OF INVENTION

This invention relates to nanoparticles for bioaffinity assays. More specifically this invention relates to ferritin particles for bioaffinity assays.

5 BACKGROUND OF THE INVENTION

The publications and other materials used herein to illuminate the background of the invention and, in particular, cases to provide additional details respecting the practice, are incorporated by reference.

10 Ferritin is a protein that is produced by e.g. bacteria, plants and mammals including humans. It is a particular and hollow protein consisting of multiple subunits that may be of different or similar type. Typically, a ferritin molecule consists of 24 subunits which self-assemble to a spherical structure. For example, human liver ferritin consists of heavy subunits (molecular weight 21 kDa) and light subunits (19 kDa) [Boyd D, Vecoli C, et. al. (1985) Journal of Biological Chemistry
15 260:11755-61]. It has diameter of 12 nm and an inside cavity with diameter of 8 nm. The inside cavity is capable of storing about 4500 ions of iron as hydrous ferric oxide [Chasteen ND, Harrison PM. (1999) Journal of Structural Biology 126:182-94]

20 In addition, ferritin-like spherical proteins with smaller size have been found in bacteria. Examples are Dpr protein produced by *Streptococci* [Haataja S, Penttinen A, et. al. Acta Crystallographica D (2002) 58:1851-1853], Dps protein produced by *Listeria* [Bozzi M, Mignogna G, et. al. (1997) Journal of Biological Chemistry 272:3259-3265], Dps protein produced by *Helicobacteria* [Tonello F, Dundon WG, et. al. (1999) Molecular Microbiology 34:238-246] and Dps protein

produced by *Escherichia* [Ilari A, Ceci P, et. al. (2002) Journal of Biological Chemistry 277:37619-37623].

The iron core is visible in electron microscopy and it has been utilised in electron microscopy as a label [Anderson KL. (1998) Biotechnic and Histochemistry
5 73:278-88].

Ferritin has been conjugated with other molecules chemically [Hsu KC. (1981) Scanning electron microscopy 4:17-26] or by protein fusions [Lofdahl, S, Uhlen, M et. al. US Pat 5,100,788].

Expression of foreign proteins or peptides on the surface of viruses as virus
10 surface protein fusions has been widely studied. The display of proteins and peptides on the surface of bacteriophages T4, T7 and λ have also been studied [Sternberg, N. & Hoess, R. (1995), Proc. Natl. Acad. Sci. USA 92, 1609-1613; Ren, Z. & Black, L. (1998), Gene 215, 439-444; Danner, S. & Belasco, J. (2001), Proc. Natl. Acad. Sci. USA 98, 12954-12959]. In these examples phages connect
15 the binding activity located on the surface of a particle to genetic information located inside the particle. Moreover, there has been research on the expression of foreign proteins on the surface of viruses with the goal of using these modified viruses as vaccines, immunogens and coating agents (US 5,008,373; US 5,041,385; US 5,463,024; US 5,736,368; US 5,804,196 and US 6,051,410).
20 Furthermore, the use of viral or phage based capsids as functional particles in bioaffinity assays has been suggested (US 2002/0025515).

Advanges of phage and viral capsids are that they self-assemble into particle like structures. They have, however disadvantages: They are replicative in their respective hosts. They can infect their hosts by accident and accordingly result in
25 uncontrolled outbreak of viruses or phages. They also contain nucleic acid molecules that may result in accidental production of unwanted proteins in unforeseen situations. The size of even the smallest bacteriophage is larger than optimal for optimal colloidal stability of the particles.

- A marker is a molecule, which is possible to detect by chemical or physical means. Marker may have catalytic activity, which is used for the detection. Examples of those markers are nucleic acids or proteins that have catalytic activity. A useful way to detect a marker is based on its fluorescence, luminescence, optical, electric or magnetic properties. Marker proteins are widely used in biological research and especially in bioaffinity assays. The following enzymes are examples of widely used marker proteins: alkaline phosphatase (EC 3.1.3.1), β -Galactosidase (EC 3.2.1.23), β -Glucuronidase (EC 3.2.1.31), glucose oxidase (EC 1.1.3.4), luciferase (EC 1.13.12.7) and horseradish peroxidase (EC 1.11.1.7). The common feature with all of them is that their detection is possible at low concentrations by using a simple protocol. In addition to enzymes, alternative marker proteins have been described, such as fluorescent proteins [Heim R, and Tsien RY, (1996), *Current Biology* 6: 178-82] or coloured proteins [Lukyanov KA, Fradkov AF, et. al. (2000), *Journal of Biological Chemistry* 275: 25879-82].
- Numerous well-defined conjugates between a binding molecule and a marker protein for assay development have been produced. A conjugate is traditionally produced by in vitro labelling of binding molecule with marker protein or peptide [Kopetzki, E.; Lehnert, K; Buckel, P. *Clin. Chem.* (1994), 40: 688-704]. An alternative way to produce the conjugate is to fuse genes encoding a binding molecule and a reporter protein, which results in the production of a fusion protein having both binding and marker activity [Zenno and Inouye, *Biochemical and Biophysical Research Communications* (1990), 171:169-74].

OBJECT AND SUMMARY OF THE INVENTION

- One object of the present invention is to provide alternative particles for bioaffinity assays.

Another object of the present invention is to provide bioaffinity assays making use of the alternative particles.

Yet another object of the present invention is to provide improved kits for bioaffinity assays making use of the alternative particles.

Thus, this invention provides a nanoparticle, useful for bioaffinity assays, comprising a self-assembling shell built up of several protein and/or peptide subunits, which protein and/or peptide subunits can be of one or several different types, assembled in an organized manner to form the shell having an inner surface facing the inside and an outer surface facing the outside of said particle wherein

- a) one or several of the types of subunits have one or several first binding moieties per type of subunit with the binding moiety facing the outside of the particle for binding of any specific ligand binding protein; and
- b)
 - i) the particle contains within its shell a marker and/or
 - ii) one or several of the types of subunits have one or several second binding moieties per type of subunit with the binding moiety facing the inside and/or the outside of the particle for binding a marker; and
- c) the marker or markers enables detection of the particle.

Characteristic for the nanoparticle is that the shell of the nanoparticle is a recombinant apoferritin or an apoferritin-like particle.

This invention also provides a bioaffinity assay using the nanoparticle.

- 20 This invention further provides a kit for a bioaffinity assay comprising the nanoparticle.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the structure of a protein nanoparticle.

Figure 2 shows detection of the binding activity of a protein particle.

- 25 Figure 3 shows the structure of plasmid pBccpHFI.

Figure 4 shows the structure of plasmid pPrGHFI.

Figure 5 shows the structure of plasmid pTSHschFI.

Figure 6 shows the structure of plasmid pCBPHFI.

DETAILED DESCRIPTION OF THE INVENTION

5 Definition of terms

The terms "apoferritin" shall be understood to consist of ferritin deficient of molecules in the cavity inside of the protein.

The term "an apoferritin-like particle" shall be understood to consist of a ferritin-like particle deficient of molecules in the cavity inside of the protein.

- 10 The "apoferritin-like particle" typically is a self-assembling particle of protein consisting of a specific number of subunits. The particle is typically spherical. Examples of ferritin-like particles have been given above.

- 15 The term "bioaffinity assays" shall be understood to include one-step and multi-step competitive and non-competitive ligand binding assays and immunoassays based on a single or multiple specific ligand binding moieties, e.g. monoclonal antibodies, polypeptides, receptors, recombinant antibodies or antibody fragments as well as artificial binders like aptamers and engineered proteins.

- 20 Bioaffinity assays include heterogeneous and homogenous assays. In heterogeneous assay the analyte is bound to a solid phase and particle is used for the detection of bound analyte. A particle can bind directly to the analyte or to a molecule, which is bound to the analyte. Particle and analyte can be added to reaction either sequentially or simultaneously. In homogenous assay the analyte is detected from solution without the separation of unbound particle. Homogenous assay can be based on for example Fluorescence Resonance Energy Transfer
25 (FRET) or Bioluminescence Resonance Energy Transfer (BRET) (Boute, N,

Jockers, R and Issad, T. 2002. Trends in Pharmacological Sciences 23:351-354). It can also be based on channeling of substrate, product or intermediate of an enzyme reaction (Gibbons, I, Armenta, R, DiNello, RK and Ullman, EF. 1987. Methods in Enzymology 136:93-103).

- 5 The term "marker" shall be understood as a feature detectable by measuring luminescence or absorbance, as well as other optical properties, electrical properties e.g. electrical current or voltage or magnetic properties, originating directly or resulted indirectly from the existence of the feature. Example of direct measurement of the feature is measurement of fluorescence emission of green
10 fluorescent protein using appropriate excitation light. Example of indirect measurement of the feature is measurement of luminescence originating from a chemical reaction catalysed by luciferase enzyme marker. Example of measurement of electrical properties is e.g. detection of redox-reaction by measuring electrical voltage or current.
- 15 The term "luminescence" shall be understood to cover luminescence, bioluminescence, chemiluminescence, electroluminescence, photoluminescence, fluorescence, delayed fluorescence and phosphorescence.

The term "luminescent protein" and "fluorescent protein", respectively, shall be understood as a protein or enzyme, which produces luminescence or is
20 fluorescent, respectively, with or without prosthetic groups. Example of luminescent protein is luciferase enzyme. Example of fluorescent protein is green fluorescent protein (GFP).

The term "nanoparticle" shall be understood as a particulate reagent composed of multiple "subunits", each composed of proteins or polypeptides and, in addition,
25 optionally of single or multiple features of the following: nucleic acids, prosthetic groups, organic and inorganic compounds. The particulate has at least a single "binding moiety" on the outer surface and the particulate contains a single "marker" or multiple "markers". Dimensions of the particulate are between one nanometer and ten micrometers.

The term "lanthanide" and "rare earth metal" shall be understood to include elements and combinations of different elements of rare earth ions from the following: neodymium (Nd), samarium (Sm), europium (Eu), gadolinium (Gd), terbium (Tb), dysprosium (Dy), Erbium (Er), ytterbium (Yb) and yttrium (Y).

- 5 The term "subunit" shall be understood as a single protein or polypeptide or complex of multiple proteins or polypeptides, composed of identical or different components.

- 10 The term "binding moiety" shall be understood to cover monoclonal antibodies, polypeptides, receptors, recombinant antibodies or antibody fragments as well as artificial binders like aptamers and engineered proteins, or derivatised form of any of the listed features. Example of polypeptide is calmodulin binding peptide (CBP). Example of derivatised feature is a peptide sequence of biotin carboxyl carrier protein (BCCP), which can be biotinylated in vivo with BirA biotin ligase enzyme.

- 15 The term "enzyme" shall be understood a protein or polypeptide or nucleic acid with catalytical activity. Examples of enzymes are lusiferase and galactose oxidase (GAO).

The term "galactose oxidase (GAO)" shall be understood as enzyme with Enzyme Commision number EC 1.1.3.9.

- 20 The term "colored protein" shall be understood as a protein or polypeptide, which has a significant absorption at visible wavelengths, 300 – 700 nm, with or without prosthetic groups.

The term "organic molecule" shall be understood as any chemical compound containing at least carbon with molecular weight below 7000 Dalton. Examples of organic molecules are prosthetic groups in fluorescent allophycocyanin protein.

- 25 The term "inorganic molecule" shall be understood as any inorganic atom, chemical compound composed of inorganic atoms or combination of atoms in an organized manner. Example of inorganic molecule is fluorescent CdSe semiconductor particle.

The term "self-assembling shell" shall be understood as a particulate structure capable of assembling itself from a pool of vital shell proteins.

The term "vital shell protein" shall be understood as protein which is needed for the self-assembly of a particulate entity e.g. nanoparticle.

- 5 The term "GFP" shall be understood as green fluorescent protein from *Aequorea victoria*, its mutant derivatives or homologous protein from other species.

The term "CRP" shall be understood as C-reactive protein.

The term "TSH" shall be understood as thyroid stimulating hormone.

The term "BCCP" shall be understood as Biotin carboxyl carrier protein.

- 10 The term "Protein G" shall be understood as Protein G from bacteria in genera *Streptococcus*.

The term "Protein A" shall be understood as Protein A from bacteria in genera *Staphylococcus*.

- 15 The term "Protein L" shall be understood as Protein L from bacteria in genera *Peptostreptococcus*.

General description of preferred embodiments

- 20 The nanoparticles according to the invention can have useful properties. These can be, but are not limited to, low cost, simple production, high stability and highly defined structure. Production can be very simple whereas a simple microbial fermentation with minor down-stream processing is typically all that is needed.

The nanoparticle according to the invention can have a marker that is an enzyme, luminescent protein, fluorescent or coloured protein or organic molecule, or a rare earth metal. If the marker is a protein, it can be an enzyme such as luciferase or GAO, or a fluorescent protein like GFP. If the marker is a rare earth metal ion, it

can be a Tb, Eu, Sm or Dy ion. The marker can also be an inorganic particle, e.g. a CdSe particle.

The nanoparticle of the invention can have, in addition to the first and second binding moieties third binding moieties. One or several of the types of subunits
5 can e.g. have one or several third binding moieties per type of subunit with the binding moiety facing the outside of the particle for binding to a solid support. The nanoparticle can also have additional binding moieties with additional functions.

The shell of the nanoparticle is a recombinant apoferritin or a ferritin-like particle.

The first, second, third or additional binding moiety can be protein A, protein G,
10 protein L or calmodulin binding peptide (CBP). The first, second, third or additional binding moiety can be an antibody against e.g. CRP, ABO blood group antigens or TSH. The first, second, third or additional binding moiety can be protein A, protein G, protein L or CBP.

The minimum radius of the nanoparticle is typically from 10 to 40 nm. The number
15 of subunits of the shell of the nanoparticle is typically more than 8, preferably more than 20.

Apoferritin has a size that results in enhanced colloidal stability. It consists only protein subunits without nucleic acids, which makes it a biosafe particle as such and it also self-assembles in solution.

20 Description of the figures

Figure 1 shows the structure of a protein nanoparticle according to the invention. The figure shows a protein shell 1, a first binding molecule 2 facing the outside of the nanoparticle, a marker 3 within the shell of the nanoparticle and a second or third binding molecule 4 facing the outside of the nanoparticle.

25 Figure 2 shows detection of binding activity of a protein particle. Analyte specific to the binding molecule to be tested is labelled with a molecule suitable for detection. Labelled analyte is then reacted with particles and particle/analyte complexes are

separated from unbound analyte by gel filtration and the signal of the label is measured.

Figure 3 shows the structure of plasmid pBccpHFI. Abbreviations stand for: Bccp-ferritin = gene encoding BCCP-Human ferritin light chain fusion protein.

- 5 Figure 4 shows the structure of plasmid pPrGHFI. Abbreviations stand for: pBR322 ori = Origin of replication, Kan = kanamycin resistance gene, LacI = gene encoding Lac repressor, Protein G-ferritin = gene encoding Protein G-Human ferritin light chain fusion protein.

- 10 Figure 5 shows the structure of plasmid pTSHscHFI. Abbreviations stand for: anti-TSHsc-ferritin = gene encoding antiTSHscFv antibody-Human ferritin light chain fusion protein.

Figure 6 shows the structure of plasmid pCBPHFI. Abbreviations stand for: CBP-ferritin = gene encoding CBP-Human ferritin light chain fusion protein.

Methods

- 15 DNA manipulations

All DNA manipulations were made according to known protocols [Sambrook J, Fritsch EF, and Maniatis T, (1989), Molecular Cloning: A laboratory manual, Cold Spring Harbour Laboratory Press, Cold Spring Harbour),

Bacteria strains

- 20 The following bacterial strains are used in the examples: XL-1 Blue, BL21, BL21(DE3), BL21(DE3:pLysS), Origami B, Origami B(DE3) and Origami B(DE3:pLysS) (Stratagene, La Jolla, CA, USA).

Measurement of fluorescence

Time-resolved fluorescence of europium and terbium was measured with Delfia reagents from PerkinElmer Life Sciences (Boston, MA, USA). Fluorescence of Alexa Fluor 594 (Molecular Probes Europe, Leiden, The Netherlands) was measured as suggested by the manufacturer. All measurements were done with Wallac Victor multilabel counter (Perkin Elmer Life Sciences, Boston, MA, USA).

Detection of reporter proteins

The activity of firefly luciferase was determined by measuring luminescence produced with Luciferase Assay Kit from BioThema Ltd (Haninge, Sweden).

10 The activity of galactose oxidase was determined by measuring luminescence from luminol oxidation by H_2O_2 generated in the reaction of enzyme with 50 mM galactose in 100 mM phosphate buffer containing 1 mM luminol and 0.4 mM $CuSO_4$ at pH 8.6. All measurements were done with Wallac Victor multilabel counter (Perkin Elmer Life Sciences, Boston, MA, USA).

15 **Example 1**

Production of ferritin based particles

Escherichia coli cells expressing plasmids encoding a binding molecule fused to N-terminus of ferritin (Example 8, Example 9, Example 10 and Example 11) subunits were grown in 50 ml of SB medium in shaking at 37 °C until OD_{600} reached 0.4. The protein production was induced by adding IPTG to the concentration of 0.5 mM and the cultivation was continued at 26 °C over night. After that the cells were collected by centrifugation at 1500 g for 10 minutes and suspended to 5 ml of phosphate-buffered saline (PBS). The cells were then lysed by sonication and cell debris removed by centrifugation at 5000 g for 10 minutes. Supernatant was then filtered with 100 kDa cut-off value filters (Pall Life Science, Ann Arbor, MI, USA) and retentate was suspended to PBS.

Example 2

Purification of the particles with gel filtration

Protein nanoparticles were purified by gel filtration with 10 ml Sepharose 6B column (Amersham Biosciences Corp, Piscataway, NJ, USA). The column was first equilibrated by using buffer containing 5 mM Tris-HCl, 0.01 % Tween-20 and 0.05 % NaN₃ at pH 7.5 with 10 volumes of the column. A 500 µl sample was applied to the column and 1 ml fractions were collected.

Example 3

Detection of binding activity on the surface of protein particle by using gel filtration

10 Rationale of the analysis of the activity of binding molecules on the surfaces of the particles is shown in Figure 2. Analyte specific to the binding molecule to be tested was labelled with a molecule suitable for detection. Analytes were labelled as follows: TSH (thyroid stimulating hormone) (Scripps Laboratories, San Diego, CA, USA), streptavidin (Perkin Elmer Life Sciences, Boston, MA, USA) and antibodies
15 with Europium and Calmodulin with Alexa Fluor 594. Mixture of four monoclonal Eu-labelled antibodies was used in the analysis of Protein G activity. Labelling of the molecules with Europium was done with a reagent kit obtained from Perkin Elmer Life Sciences, (Boston, MA, USA). Labelled calmodulin was obtained from Molecular Probes Leiden, The Netherlands). Labelled analyte was reacted with
20 particles and particle/analyte complexes were separated from unbound analyte by gel filtration as described in Example 2. Ferritin based particles were produced as described in Example 1. Particles produced without binding molecule were used as negative control.

Example 4Detection of binding activity on the surface of protein particle by using multiwell plates

- 5 In vitro biotinylation of TSH and antibodies was done by reagent kit obtained from Perkin Elmer Life Sciences, Boston, MA, USA). Mixture of four monoclonal Eu-labelled antibodies was used in the analysis of Protein G activity. Biotinylated molecules were attached to streptavidin-coated 96 well plates (Innotrac Diagnostics, Turku, Finland) as described in the kit and wells were washed four times. After that the particles to be tested were added and the wells were again
- 10 washed for four times. Then the analyte labelled as described in Example 3 was added, wells washed four times and signal from the label measured.

Example 5Detection of binding activity on the surface of protein particle by using molecular weight cut-off filters

- 15 Analyte molecules were labelled as described in Example 3. Particles together with bound analyte were separated from smaller molecules with 100 kDa cut-off value filters (Pall Life Science, Ann Arbor, MI, USA). Signal of the labelled molecule was measured from the retentate.

Example 6

- 20 Loading of ferritin based particles with terbium

The reaction buffer used in the loading of terbium ions into ferritin consisted of 50 mM HEPES, 50 mM NaCl and 10 mM TbCl₃ at pH 7.0. Ferritin was added to the buffer to 0.1 µM and the reaction was incubated at 37 °C for 20 h. Unreacted

terbium was removed by gel filtration with NAP-5 column (Amersham Biosciences Corp, Piscataway, NJ, USA).

Example 7

Loading of ferritin with europium.

- 5 Pelleted fraction of a 100 ml of bacterial culture produced according to Example 1 by using *E. coli* BL21(DE3:pLysS:pBccpHFI) was dissolved to 1.5 ml of 8 M urea and centrifuged at 5000 g. 10 ml of solution containing 100 mM Tris, 150 mM NaCl at pH 8.5 was added and the mixture was incubated at +4 °C over night. HCl was then added until pH of the solution reached 6.0 and the solution was incubated at
- 10 4 °C over night. 0,056 ml of 0.01 M EuCl_3 solution was added and solution was incubated at 25 °C for 2 h after which buffer (133 mM Tris, 150 mM NaCl, pH 9.0) was gradually added until pH reached 8.5. Precipitated europium was then separated from soluble europium-loaded ferritin by collecting supernatant after 10 min centrifugation at 5000 g. Particles loaded with europium were tested for the
- 15 binding capacity according to Example 4 and the europium inside ferritin was detected. The reaction containing excess of soluble biotin (0.001 mM biotin) was used as control. Signal without biotin was 163 fold as compared to control showing the binding of Eu labelled particles to streptavidin coated solid phase.

Example 8

20 Production of Protein G on the surface of ferritin

- Gene encoding Streptococcal Protein G was inserted to plasmid producing human ferritin light chain by ligating *NheI* digested fragment obtained by PCR with oligonucleotides 5'-AAGGATCCCATATGAACCTCTGTAACCATTTTCAG (SEQ ID NO: 1) and 5'-AACCATGGCATATGGTGACAACTTACAACT (SEQ ID NO: 2)
- 25 with *Streptococcus* G148 genomic DNA as template with *NheI* digested plasmid pET-26(+)*rHuLFt* (Grace JE Jr, Van Eden ME, Aust SD. 2000. Archives in

Biochemistry and Biophysics 384:116-22). The resulting construct was transformed to *E. coli* BL21(DE3:pLysS) cells. Structure of the resulting plasmid, pPrGHFI, was verified by partial sequencing. The structure of pPrGHFI is shown in Figure 4. Ferritin based particles expressing Protein G on their surface were produced with *E. coli* BL21(DE3:pLysS:pPrGHFI) cells by using protocol described in Example 1. The retentate was suspended to 1 ml and diluted tenfold prior to the analysis of the functionality according to Example 5 using Eu-labelled antibodies as label. The particles with Protein G gave 5.5 fold signal as compared to particles produced with pET-26(+)-rHuLFt.

10 Example 9

Production of scFv fragment on the surface of ferritin

Gene encoding anti-TSHscFv fragment was inserted to plasmid producing human ferritin light chain by ligating NheI digested fragment obtained by PCR with oligonucleotides 5'-GTTATATCAACTGTAAAAGT (SEQ ID NO: 3) and 5'-AAC-CATGGCATATGGAAATTGTGCTCACCCA (SEQ ID NO: 4) with pTSHscHoc as template with NheI digested plasmid pET-26(+)-rHuLFt (Grace JE Jr, Van Eden ME, Aust SD. 2000. Archives in Biochemistry Biophysics 384:116-22). The resulting construct was transformed to *E. coli* Origami B(DE3:pLysS) cells. Structure of the resulting plasmid, pTSHscHFI, was verified by partial sequencing. The structure of pTSHscHFI is shown in Figure 5. Ferritin based particles expressing anti-TSHscFv antibody on their surface were produced with *E. coli* Origami B(DE3:pLysS:pTSHHFI) cells by using protocol described in Example 1. The retentate was suspended to 1 ml and diluted fivefold prior to the analysis of the functionality according to Example 5 using Eu-labelled TSH as label. The particles with anti-TSHscFv gave 33-fold signal as compared to particles produced with pET-26(+)-rHuLFt.

Example 10

Production of Calmodulin Binding Peptide on the surface of ferritin

Gene encoding cbp was constructed in two PCR reactions. First PCR was done with oligonucleotides 5'-GAATTCGGATCCTTAGTCGTGCTTG (SEQ ID NO: 5) and 5'-CTGCTGCGAACCGTTTCAAGAAAATCAGCTCTTCCGGTGCTGGCG-GTATGAGCTCCCAGATTCGTCAGAATT (SEQ ID NO: 6) with pET-26(+)-rHuLFt as template. The product of the first PCR was used as template of the second PCR with oligonucleotides 5'-GAATTCGGATCCTTAGTCGTGCTTG (SEQ ID NO: 7) and 5'-TAGATATACATATGAAACGCCGTTGGAAGAAAGCGTTCATCG-CTGTTTCTGCTGCGAACCGTTTCAAGAAAAT (SEQ ID NO: 8). The NdeI-BamHI digested second product was ligated with 5.3 kb NdeI-BamHI fragment of pET-26(+)-rHuLFt. The resulting construct was transformed to *E. coli* BL21(DE3:pLysS) cells. Structure of the resulting plasmid, pCBPHFI, was verified by partial sequencing. The structure of pCBPHFI is shown in Figure 6. Ferritin based particles expressing cbp on their surface were produced with *E. coli* BL21(DE3:pLysS:pCBPHFI) cells by using protocol described in Example 1. The retentate was suspended to 1 ml and diluted five-fold prior to the analysis of the functionality according to Example 3 with Alexa Fluor 594 labelled calmodulin. The particles with cbp gave 4.1-fold signal as compared to particles produced with pET-26(+)-rHuLFt.

Example 11

Production of Biotinylated Peptide on the surface of ferritin

Gene encoding Biotin Carboxyl Carrier Peptide (BCCP) was inserted to plasmid producing human ferritin light chain by ligating NheI digested fragment obtained by PCR with oligonucleotides 5'-AACCATGGCATATGGAAGCGCCAGCAGCAGC (SEQ ID NO: 9) and 5'-AGCCGCTGGTCGTCATCGAGGGTGGCCATATGCGTT-GCAA (SEQ ID NO: 10) with *E. coli* XL-1 Blue genomic DNA as template with

NheI digested plasmid pET-26(+)*rHuLFt* (Grace JE Jr, Van Eden ME, Aust SD. 2000. Archives in Biochemistry and Biophysics 384:116-22). The resulting construct was transformed to *E. coli* BL21(DE3:pLysS) cells. Structure of the resulting plasmid, pBccpHFI, was verified by partial sequencing. The structure of pBccpHFI is shown in Figure 3. Ferritin based particles expressing BCCP on their surface were produced with *E. coli* BL21(DE3:pLysS:pBccpHFI) cells by using protocol described in Example 1. The retentate was suspended to 1 ml and diluted thousand fold prior to the analysis of the functionality according to Example 4 using Eu-labelled streptavidin as label. The particles produced with pBccpHFI gave 326 fold signal as compared to particles produced with pET-26(+)*rHuLFt*.

It will be appreciated that the present invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent for the specialist in the field that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

CLAIMS

1. A nanoparticle, useful for bioaffinity assays, comprising a self-assembling shell built up of several protein and/or peptide subunits, which protein and/or peptide subunits can be of one or several different types, assembled in an organized manner to form the shell having an inner surface facing the inside and an outer surface facing the outside of said particle wherein
 - a) one or several of the types of subunits have one or several first binding moieties per type of subunit with the binding moiety facing the outside of the particle for binding of any specific ligand binding protein; and
 - 10 b) i) the particle contains within its shell a marker and/or
 ii) one or several of the types of subunits have one or several second binding moieties per type of subunit with the binding moiety facing the inside and/or the outside of the particle for binding a marker; and
 - c) the marker or markers enable detection of the particle;
- 15 **characterised** in that the shell of the nanoparticle is a recombinant apoferritin or a an apoferritin-like particle.
2. The nanoparticle according to claim 1 **characterised** in that the marker is selected from the group consisting of an enzyme, luminescent protein, fluorescent or coloured protein or organic molecule and a rare earth metal.
- 20 3. The nanoparticle according to claim 2 **characterised** in that the marker is a protein, optionally an enzyme, selected from the group consisting of luciferase, GAO and GFP.
4. The nanoparticle according to claim 2 **characterised** in that the marker is a lanthanide preferably selected from the group consisting of Tb, Eu, Sm and Dy.
- 25 5. The nanoparticle according to claim 1 **characterised** in that one or several of the types of subunits have one or several third binding moieties per type of subunit

with the binding moiety facing the outside of the particle for binding to a solid support.

6. The nanoparticle according to any of preceding claims **characterised** in that a first binding moiety is selected from the group consisting of protein A, protein G,
5 protein L, calmodulin binding peptide (CBP) and biotin carboxyl carrier protein (BCCP).
7. The nanoparticle according to any of claims 1 to 6 **characterised** in that a first binding moiety is an antibody against one of members of the group consisting of CRP, ABO blood group antigens and TSH.
- 10 8. The nanoparticle according to any of claims 1 to 6 **characterised** in that a second binding moiety is a binding moiety selected from the group consisting of protein A, protein G, protein L, calmodulin binding protein (CBP) and biotin carboxyl carrier protein (BCCP).
9. The nanoparticle according to any of claims 1 to 6 **characterised** in that a
15 second binding moiety is an antibody against one of the group consisting of CRP, ABO blood group antigens and TSH.
10. The nanoparticle according to any of preceding claims **characterised** in that the minimum radius of the nanoparticle is from 10 to 40 nm.
11. The nanoparticle according to any of preceding claims **characterised** in that
20 the number of subunits is more than 8, preferably more than 20.
12. Use of a nanoparticle according to any of preceding claims in a bioaffinity assay.
13. Kit for an immunoassay comprising the nanoparticle according to any of claims 1 to 9.

ABSTRACT

Thus this invention relates to a nanoparticle, useful for bioaffinity assays. The nanoparticle has a self-assembling shell built up of several protein and/or peptide subunits, which protein and/or peptide subunits can be of one or several different types, assembled in an organized manner to form the shell having an inner surface facing the inside and an outer surface facing the outside of said particle. One or several of the types of subunits have one or several first binding moieties per type of subunit with the binding moiety facing the outside of the particle for binding of any specific ligand binding protein; and the particle contains within its shell a marker and/or one or several of the types of subunits have one or several second binding moieties per type of subunit with the binding moiety facing the inside and/or the outside of the particle for binding a marker; and the marker or markers enables detection of the particle. Characteristic for the nanoparticle is that the shell of the nanoparticle is a recombinant apoferritin or an apoferritin-like particle. This invention also relates to a bioaffinity assays using the nanoparticle. This invention further relates to a kit for bioaffinity assays comprising the nanoparticle.

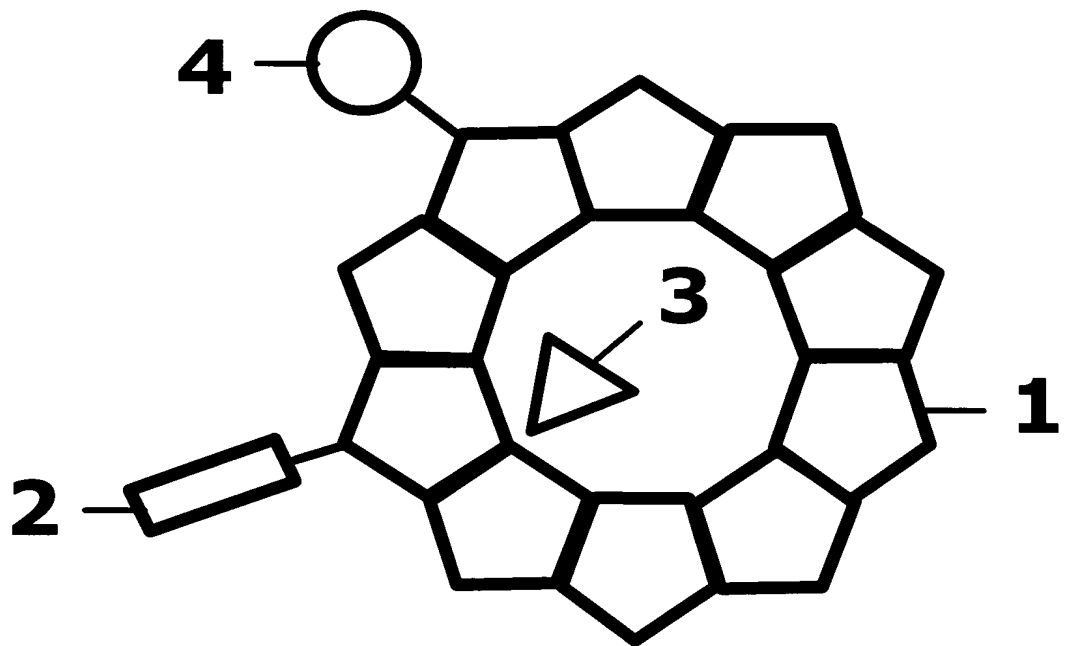
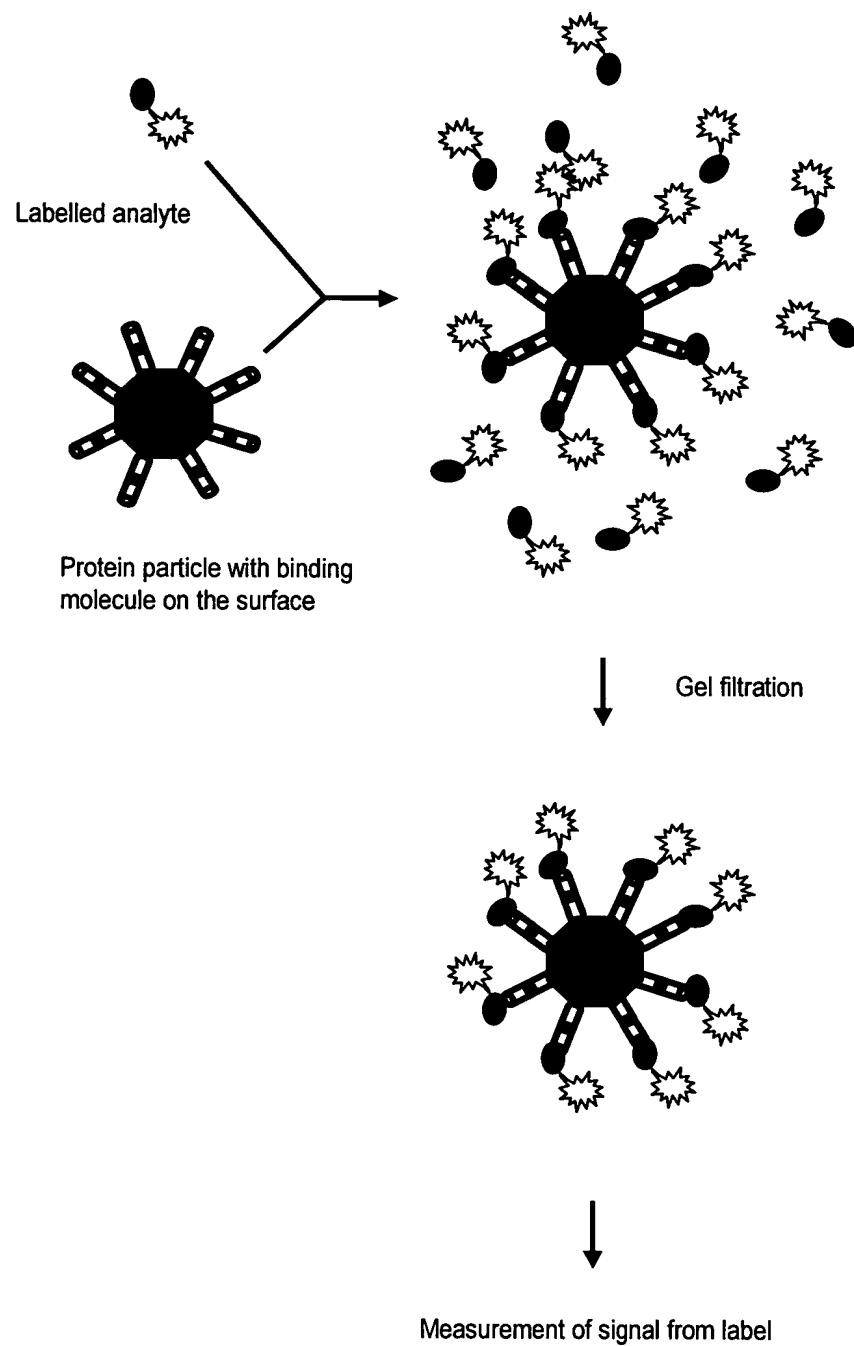
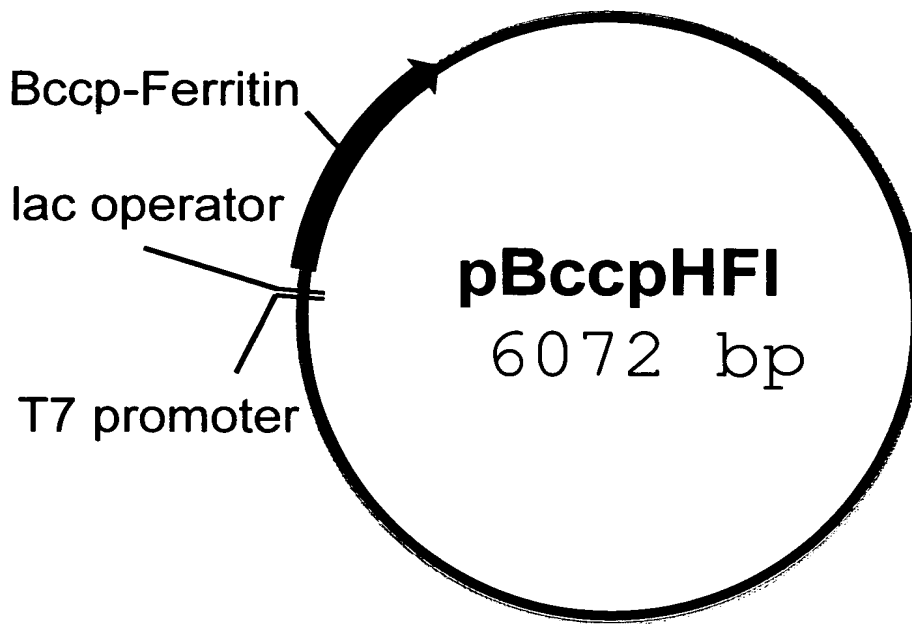
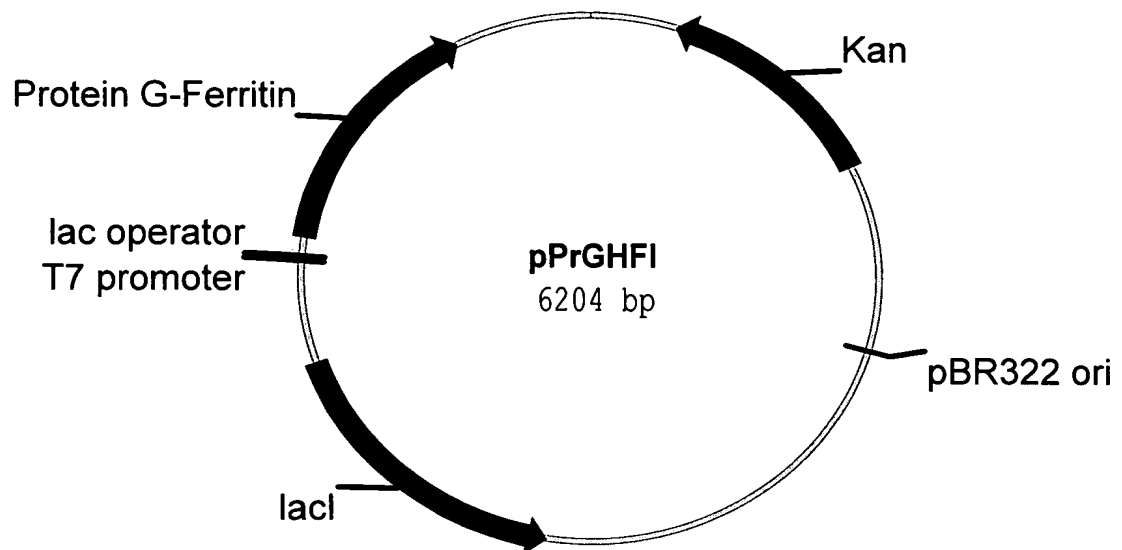


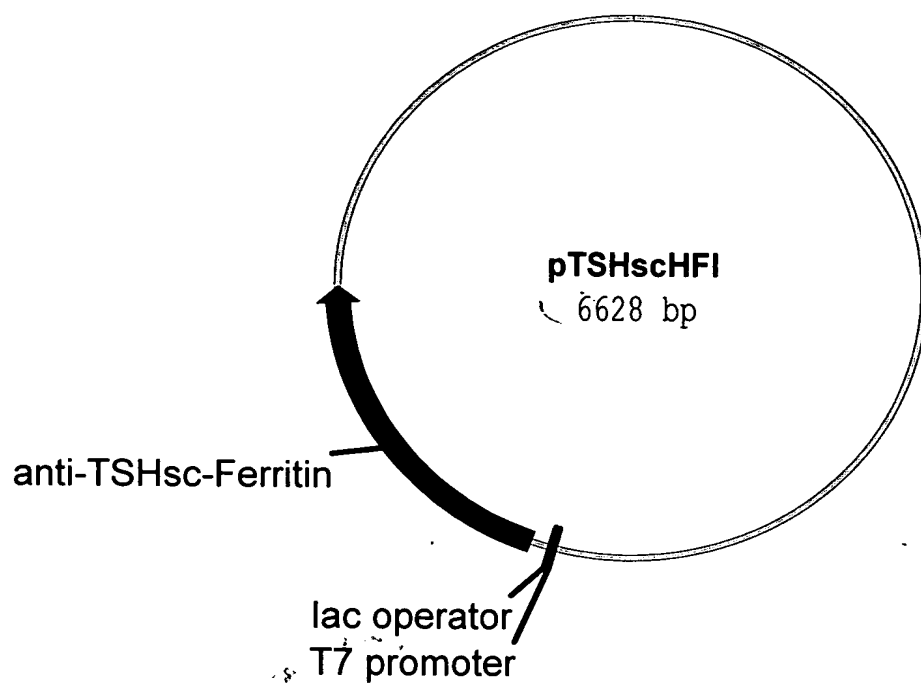
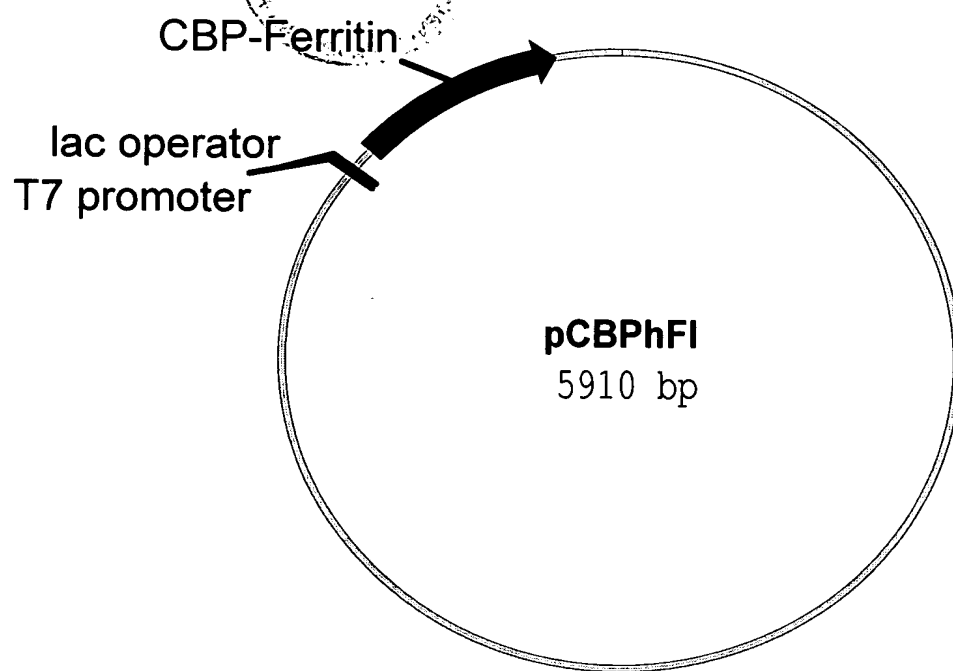
Figure 1

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**Figure 2**

**Figure 3****Figure 4**

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**Figure 5****Figure 6**

INTERNATIONAL SEARCH REPORT

International Application No

PCT/FI2004/000204

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 G01N33/543 G01N33/58

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LUZZAGO A ET AL: "ISOLATION OF POINT MUTATIONS THAT AFFECT THE FOLDING OF THE H CHAIN OF HUMAN FERRITIN IN ESCHERICHIA-COLI" EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL, vol. 8, no. 2, 1989, pages 569-576, XP002286331 ISSN: 0261-4189 the whole document figure 3	1-3,5, 10-13
X	US 5 358 722 A (MONZYK BRUCE F) 25 October 1994 (1994-10-25) the whole document claims 1,4	1-5, 10-13
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Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- * & * document member of the same patent family

Date of the actual completion of the international search

30 June 2004

Date of mailing of the international search report

26/07/2004

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI2004/000204

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

a. type of material

☒

a sequence listing

☐

table(s) related to the sequence listing

b. format of material

☒

in written format

☒

in computer readable form

c. time of filing/furnishing

☒

contained in the international application as filed

☒

filed together with the international application in computer readable form

☐

furnished subsequently to this Authority for the purpose of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: